VITAMIN EFFECTS IN THE PHYSIOLOGY OF MICROORGANISMS

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No sooner had vitamins been discovered to be essential in the growth and reproduction of certain of the higher animals than similar claims were put forth in regard to their importance in the physiology of microörganisms. Confusion as to the identity of bios and vitamin B led to further unjustifiable extensions of the meaning of the term vitamin until substances acting as growth or reproductive stimulants were included in the category of vitamins, although wholly unnecessary for the processes of growth and reproduction themselves. In fact as Fulmer (1922) pointed out it has been attempted on the basis of just such stimulation to ascribe entirely new properties to vitamins in direct conflict with the only adequate test now known—the effect on the growth and reproduction of a suitable animal.

Substances of the nature of vitamin B have been claimed to be essential or markedly stimulating to the growth and reproduction of various microörganisms. Only a few of the more pertinent references will be cited here and the reader is referred to Tanner's review of the bios question for a comprehensive resumé (1925).

Linossier (1919, 1920) reported that *Oöspora lactis* could grow in synthetic media but that it was sensitive to the addition of vitamins. The addition of vitamin extracts markedly increased the crop. Sammartino (1921) reported that both rapidity of reproduction and rapidity of fermentation of *Saccharomyces cerevisiae* are affected by vitamins.

Funk and Friedman (1922) found for both yeasts and bacteria growth stimulating substances which they claimed to be of a vitamin nature, present in beef, pepton, beef heart infusions and autolyzed brewer's yeast. Suzuki and Suzuki (1923) separated from vitamin B by means of aluminum cream a substance promoting bacterial growth, which they called vitamin D and stated that it was a decomposition product of vitamin B. Broadhurst (1921) reported remarkable revivification of old bacterial cultures by use of an aqueous extract of the navy bean. She attributed the results to vitamins.

Itano (1923) in a study of the influence of vitamin B upon the rate of growth of *Azotobacter chroococcum* concluded that it exerted a marked stimulating influence on reproduction and nitrogen fixation. Referring to vitamin B concentrate and nucleic acid Itano concluded that "it seems to be apparent that these two substances exert an accessory influence other than the supply of a very small quantity of food substance." He believed his experimental data indicated plainly that the addition of vitamin B to the medium had a stimulating influence on *Azotobacter chroococcum.*

Sanborn (1926) in a recent article concluded that "the essential food factor represented by vitamin B (?) exerts a stimulating effect upon the growth and physiological efficiency of *C.* (*Cellulomonas*) *folia.*" In the work of Itano and of Sanborn a vitamin concentrate was added to the medium to supply vitamin B.

The numerous investigations purporting to indicate a stimulative effect of vitamin B on bacterial metabolism show a general disregard of the effect of small and easily available quantities of food present during the lag phase on the bacterial numbers occurring during the phase of logarithmic reproduction. A similar disregard of the possible effect of the vitamin synthesized by the microörganism is manifested.

In a former communication (1925) it was shown that numerous species of bacteria, yeasts and fungi may be continuously cultured in wholly synthetic media employing inorganic salts and either methose, or synthetic succinic acid, as sources of energy. In the present paper it is shown that the addition of vitamin B to the medium does not stimulate reproduction of certain nitrogen fixing organisms, particularly *Azotobacter chroococcum.* It is shown that the addition of a vitamin B *concentrate* may afford a transient stimulation which is not of a type correctly ascribed to vitamins and is incidentally the result of circumstances in which vitamin B plays no essential part. It is shown that stimulation due to a small quantity of food substance other than vitamin B may be differentiated from effects ascribable to vitamins by determining the rates of multiplication of an organism.

EXPERIMENTAL

Effect of vitamin B on the rate of multiplication of Azotobacter chroococcum and Rhizobium leguminosarum

The vitamin B concentrate used was prepared by the Harris Laboratories. The preparation was advertised as containing vitamin B in concentrated form (Osborne and Wakeman, fraction 2). Rat tests indicated that the material was rich in vitamin B. Its nitrogen content by the Kjeldahl method was 0.75 mgm. per gram of concentrate. It was tested for its action on the rate of multiplication of microörganisms by adding to Ashby solution of pH 7.2. This medium contained only chemically pure salts and sucrose. As a precaution against the possible presence of vitamin B, the sucrose was extracted continuously for seventy-two hours with hot 95 per cent alcohol. A 1 per cent solution of the concentrate was prepared and varying amounts added to double strength Ashby solution. Sterile distilled water was then added to volume. The medium was tested for sterility before use. Equal quantities of a twenty-four to forty-eight hour culture of *Azotobacter chroococcum* or *Rhizobium leguminosarum* were added as inoculum.

All culture flasks in an experiment were connected by means of a manifold to a vacuum line and the number of bubbles of air passing through controlled by means of screw clamps. A current of air sufficient to maintain a circulation of the medium was employed. Cotton plugs in the inlets and outlets prevented contamination; an acid tower prevented absorption of atmospheric ammonia by the cultures. Reproduction of Azotobacter and Rhizobium is markedly stimulated by aeration due to a better distribution of oxygen and nitrogen throughout the medium. The range of growth in the medium is greatly extended, that is, growth and reproduction occur throughout the medium and not simply near the surface as occurs in unaerated liquid cultures.

Plate counts were abandoned since any strain of Azotobacter or Rhizobium forms some gum and plate counts were found unreliable. Direct counts were made by use of a Helber counting chamber.

Stimulation of bacterial multiplication may best be determined by comparing velocity coefficients of the rate of multiplication. This is most conveniently performed for the logarithmic phase. Our classification of the life phases in a bacterial culture is that suggested by Buchanan (1918). During the logarithmic phase the rate of multiplication is constant. The velocity coefficient (k) is determined by the relationship

$$k = \frac{\log b - \log B}{t}$$

in which k is the velocity coefficient, i.e., the rate of reproduction per cell, t is time in hours, b is the number of bacteria present after time t, and B the number of bacteria present when t is equal to 0. Graphical representation of results may be accomplished by plotting logarithms of the numbers of bacteria as ordinates against times in hours as abscissae. Since the rate of multiplication during the logarithmic phase remains constant, the curve during this period is represented by a straight line. The greater the slope of this line the greater the rate of multiplication. We may, therefore, compare graphically the rates of multiplication of two cultures during the "logarithmic" phase by comparing the slopes of these lines. Since a straight line represents the rate of multiplication, coefficients may be determined for any interval of time during this phase. However, the entire line was employed to determine velocity coefficients because it was found that more accurate results were obtained in this manner.

Experimental determination of a mathematical expression of the degree of stimulation occurring during the lag phase is more difficult since there is an acceleration of the rate of multiplication per cell which approaches the constant of the logarithmic phase. A time measurement of lag may be of value in these experiments since all cultures were started with equivalent inoculations.

Table 1 gives the results of one of numerous experiments and affords data which are subject to analysis. In a 1:10,000 concentration, the vitamin *concentrate* evidently induces a definite increase in the number of organisms present during the early

TABLE 1

Effect of vitamin B on the rate of reproduction of Azotobacter

FLASK NUMBER	TREATMENT (ASHBY MEDIUM)	ORGANISMS, MILLIONS PER CUBIC CENTIMETER AFTER HOURS AT 25°C.							k
		0 hours	6 hours	12 hours	18 hours	30 hours	42 hours	54 hours	
1	Vitamin concentrate 1:1,000	2.0	4.0	8.3	16.0	58	230	916	0.872
2	Vitamin concentrate 1:10,000	2.0	3.5	6.4	14.0	46	190	750	0.881
3	Vitamin concentrate 1:1,000,000	2.0	2.2	3.0	4.6	19	78	300	0.872
4	Alcohol extract equivalent to 1,100,000 concentrate	2.0	2.2	2.9	4.9	18	84	300	0.860
5	Extracted concentrate 1:1,000	2.0	3.9	7.6	14.0	50	210	860	0.915
6	Plain Ashby control	2.0	2.3	3.4	6.5	23	91	320	0.835

stages of growth. This holds for both *Azotobacter chroococcum* and *Rhizobium leguminosarum* (table 1).

In figure 1 are shown for more critical analysis curves for the multiplication of *Azotobacter chroococcum*. Logarithms of the numbers of bacteria are plotted as ordinates, times in hours are plotted as abscissae. An analysis of the curves indicates the nature of the apparent stimulation. It is shown that stimulation due to small quantities of food substances other than vitamin B may be differentiated from effects ascribable to vitamins by determining rates of multiplication of the organisms. Cultures to which the vitamin B concentrate was added showed a greater average acceleration—a progressively shorter average generation time—during the period of lag than did the control organisms forced to fix atmospheric nitrogen. Lag periods of ten to fifteen hours were common in the control flasks. On the other hand the addition of vitamin B concentrate in 1:1000 concentration re-

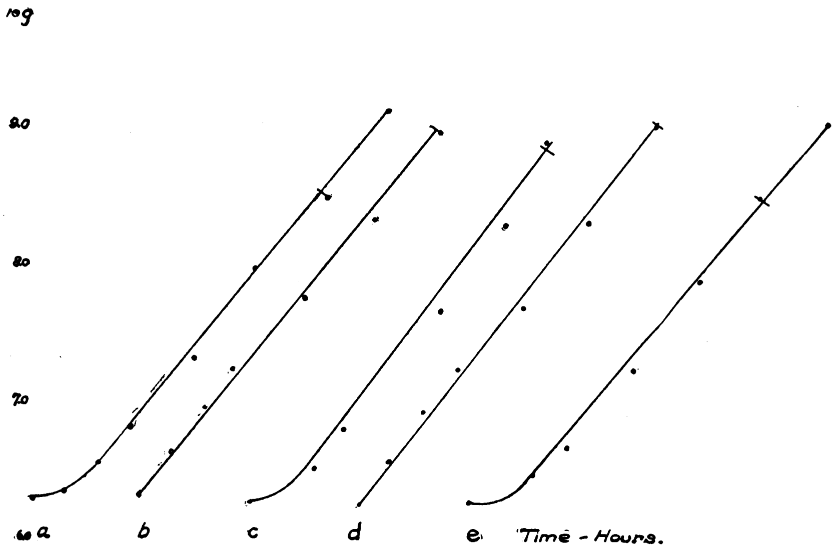

FIG. 1

A. Culture in Ashby medium (control).
B. Culture in Ashby medium plus 1:10,000 vitamin B concentrate.
C. Culture in Ashby medium plus 1:1,000,000 vitamin B concentrate.
D. Culture in Ashby medium plus 1:10,000 alcohol extracted vitamin B concentrate.
E. Culture in Ashby medium plus alcohol extract of vitamin B concentrate equivalent to 1:10,000 concentrate.

duced the lag to a period of an hour or less. It is difficult to say whether the initial stationary phase is shortened by the vitamin concentrate or whether the rate of reproduction is simply greater during the lag phase. It has seemed by using old cultures of Azotobacter (i.e., cells capable of showing an extended initial stationary phase) that both a reduction of the initial period and an increased rate of reproduction are caused by the addition of the vitamin concentrate.

Following this initial acceleration in the cultures containing the vitamin concentrate the curves become parallel and the average rates of reproduction—the average generation times in the control and vitamin cultures—are equal. That is, the cells in the original inoculum and their progeny for several generations show the stimulative action of the concentrate while cells in following generations show no such effect.

This is clearly not a vitamin effect. There is no reason to assume that the first generations would be stimulated by vitamin B while the later generations would not. A vitamin effect should be indicated by an increase in the rate of multiplication extending throughout the entire range of reproduction—at least through the lag phase and logarithmic phase. The curve during the logarithmic phase for cells receiving a vitamin stimulation would be expected constantly to ascend more rapidly than the control curve. This is apparently not the case.

Against these conclusions it might be claimed that the vitamin is used up during the lag phase and that the stimulation due to the vitamin must necessarily cease therefore. Furthermore, it is conceivable that destruction of the vitamin may occur as the result of the action of bacterial products of metabolism. Investigators claiming to have shown vitamin stimulation have tacitly assumed no such induced unavailability of the vitamin, since none of them have restricted their claims to the lag phase. In fact the nature of their experiments is assurance that their results were determined from multiplication occurring during advanced life phases. In view of the fact that a concentrated form of vitamin material was added, it is hardly conceivable that a few million bacteria could possibly use up all of the vitamin. Furthermore, we know that old cultures of Azotobacter have vitamin B available for the growth of rats. Our results with alcohol extracts of vitamin concentrate and extracted concentrate show a definite correlation of the rate of multiplication with the quantity of nitrogenous material and not with vitamin content. This point will again be considered.

If on the other hand we are dealing with a nutritional stimulation due to the presence of a small quantity of an easily available source of some essential element such as nitrogen or due to an easily available source of energy, such stimulation would be apparent only so long as the source of supply lasted. Therefore, we may determine experimentally, velocity coefficients of the rate of multiplication during the logarithmic phases of cultures of organisms with and without vitamin B added to the medium. If the addition stimulates reproduction, the coefficient, determined in the presence of vitamin B will be greater. The only other alternative is proof of the destruction or induced unavailability of the vitamin.

The utilization of a very small quantity of food material other than vitamin B by the organisms constituting the original inoculum, resulting in a greater acceleration than is shown by organisms forced to fix atmospheric nitrogen, is important in determinations purporting to show stimulation ascribable to vitamins. This point has been generally disregarded.

An hypothetical case may serve as an illustration. Culture tubes A and B containing Ashby's medium are inoculated with equal numbers of Azotobacter cells. Tube B receives an addition of a small quantity of a substance containing readily available nitrogen. It is necessary for the organisms in tube A to obtain their nitrogen from the atmosphere (a less available source than is afforded by the substance added to tube B). As a result, the organisms planted in tube B multiply more rapidly and are, let it be assumed, ten times as numerous per unit volume as in tube A at the time each enters the logarithmic phase. At this time the source of nitrogen available in tube B as the result of the addition of the nitrogenous material to the medium, becomes exhausted. Both cultures must now obtain their nitrogen from the atmosphere. Disregarding any environmental adaptations of the organisms and recalling that multiplication is geometric, we shall find (at comparable times during the logarithmic phases in the cultures) always ten times as many organisms in tube B as are in tube A. In fact, this is just the result to be expected if the rates of multiplication per cell are equal. Resort to an hypothesis of vitamin stimulation is not only superfluous but involves an erroneous interpretation of results.

An experimental demonstration of the above hypothesis is shown in figure 1. It is seen that after an initial stimulation of the rate of multiplication in the vitamin tubes, all rates are later equivalent (slopes parallel) although there are many more organisms in the vitamin B culture per cubic centimeter than in the control. The rate of multiplication per cell (k) for organisms having access to vitamin B in 1:10,000 concentration is 0.881, for controls 0.835 (table 1). At 1:10,000 dilution the concentrate contains sufficient easily available nutritional matter of a nature other than vitamin B (i.e., nitrogen) to afford these organisms an initial stimulation that carries them into the phase of logarithmic reproduction in the culture. The effect is then exhausted and stimulation ceases. The effect is purely that of providing the organisms with a better medium than is afforded by Ashby medium.

Itano (1923) and Sanborn (1926) disregard the significance of the food substances other than vitamin B in the concentrate. Itano states that fraction 2 contains 7.5 per cent nitrogen, the same as found by us but we cannot agree that this quantity is insignificant, since in a 1:10,000 concentration there would be present in 100 cc. of the medium used 0.75 mgm. of nitrogen.[1] Assuming a bacterial cell to be 85 per cent water and 15 per cent solid matter, 90 per cent of the solid matter to be protein, and 16 per cent of protein to be nitrogen, 0.75 mgm. of nitrogen would suffice for approximately 17 billions of bacteria of average size and weight (0.000,000,002 mgm.) if all the nitrogen were available for cell synthesis.

To test this assumption *Bacterium coli* was inoculated into a medium consisting of Harris vitamin B concentrate and distilled water, and into the same medium to which had been added 1 per cent glucose as a source of energy, and 0.2 per cent dipotassium phosphate. That vitamin B concentrate supplies all the elemental and energy needs of an organism is obvious from

[1] Itano states that in a 1:10,000 concentration of vitamin B concentrate which was used by him in 100 cc. quantities of medium there would be present .0075 mgm. of nitrogen. This is apparently an erroneous conclusion.

table 2. In a medium consisting only of 1:10,000 concentration of vitamin B concentrate *Bact. coli* was afforded all the elemental matter and energy requisite for the development of at least 40 millions of bacteria per cubic centimeter. If a source of energy were supplied as is the case in experiments using Ashby's medium at least 63 millions of bacteria per cubic centimeter developed. Itano and Sanborn disregard the effect of that part of the concentrate other than vitamin B and comprising the great bulk of the material and ascribe the stimulation to vitamin B.

TABLE 2
Multiplication of Bacterium coli in medium containing vitamin B concentrate as source of nutriment

FLASK NUMBER	MEDIUM 1 PER CENT DEXTROSE, 0.2 PER CENT K_2HPO_4 PLUS CONCENTRATE	ORGANISMS AFTER HOURS AT 25°C. (MILLIONS PER CUBIC CENTIMETER)		
		0 hours	24 hours	48 hours
1	1:1,000	0.25	35.0	740
2	1:10,000	0.26	13.0	63
3	1:100,000	0.24	4.0	18
4	1:1,000,000	0.25	1.1	3
5	1:1,000 concentrate, as sole medium	0.29	27	480
6	1:10,000 concentrate as sole medium	0.27	11.0	40
7	1:100,000 concentrate as sole medium	0.26	0.3	11.0
8	1:1,000,000 concentrate as sole medium	0.24	0.03	0.2
9	Distilled water only	0.27	0.20	0.2

Inasmuch as vitamin B is soluble in alcohol, if the vitamin itself is effective in stimulating growth, a very small quantity of an alcoholic extract of the concentrate should be adequate to demonstrate the effect. The following experiments reveal no vitamin stimulation when such extracts were substituted for aqueous solutions of the vitamin B concentrate.

A weighed quantity of concentrate was continuously extracted with hot 95 per cent alcohol for twenty-four hours when the alcohol was evaporated *in vacuo* until 0.1 cc. represented the extract from 0.01 gram of concentrate. This material contained small amounts of alcohol soluble nitrogenous compounds, approximately 0.1 mgm. per 0.1 cc. of extract which represented 0.01 gram of concentrate. This quantity when added to a liter of medium represented a concentration of 1:10,000 of concentrate. No stimulation due to food substance was detectable in this concentration. It is obvious from the curves and table 1 that no stimulation ascribable to vitamins was present. It is reasonable to expect a vitamin effect to be apparent under these conditions if such an effect exists.

Furthermore, it is reasonable to expect, if vitamin exerts no stimulation of bacterial multiplication, that alcohol extracted concentrate would exert the same type of stimulation as that observed in the case of unextracted concentrate. It is possible that the alcohol used in extracting would remove desirable proteins, carbohydrates or other substances along with vitamin B. A very small quantity of material is removed but this removal did not detectably influence the results of the experiments. When comparing the extracted and unextracted material, the nitrogen contents were made equivalent. This required 0.0125 gram of extracted per 0.01 gram of unextracted material.

In table 1 is shown the stimulation resulting from the addition of vitamin concentrate continuously extracted for three weeks with hot 95 per cent alcohol. The same stimulation occurs as with the unextracted concentrate.

Waksman and Skinner (1926) have recently demonstrated the stimulation afforded to cellulose fermenters by the addition of available nitrogen to the medium. They found that the amount of cellulose which is decomposed in the soil under aerobic conditions is dependent upon the amount of available nitrogen and that aerobic decomposition of cellulose sets in very early if available nitrogen is present. Niklewski (1912), Charpentier (1921), Barthel and Bengston (1924) and Waksman and Heukelekian (1924), all found that the decomposition of cellulose in the soil is largely controlled by the amount of available nitrogen. Sanborn (1926) apparently ignored the influence of the nitrogen present in the vitamin B concentrate and has failed to differentiate between such a purely nutritional stimulation due to the presence of essential elements or sources of energy in a readily available form and a vitamin stimulation which would effect a continuously greater *rate* of reproduction than occurs in the controls. He did not determine rates of reproduction, but used bacterial counts to indicate rate, a procedure which has been shown not to be justified since the initial number of bacteria present, or present soon after as the result of an initial and temporarily more favorable medium, determines the numbers present at a later stage.

There can be only one possible stimulation of bacterial reproduction by vitamin B and that is a stimulation due to the vitamin B synthesized by the organism itself and such a stimulation would not be apparent in any of the experiments discussed above, since the controls themselves would be subject to the same effect.

In fact both the above organisms do elaborate vitamin B (unpublished results) and any determination of a vitamin B stimulation would be rendered rather hazardous by this fact.

CONCLUSIONS

1. The addition of vitamin B to Ashby medium exerts no stimulation of the rate of reproduction of *Azotobacter chroococcum* or *Rhizobium leguminosarum*.

2. The addition of vitamin B concentrate (Harris) to Ashby medium stimulates the rate of multiplication of *Azotobacter chroococcum* and *Rhizobium leguminosarum*, as the result of adding readily available nutrients other than vitamin B (i.e., nitrogen or source of energy). The purely nutritive effect has been confused with a vitamin effect.

3. Until further knowledge is at hand, the meaning of the term vitamin should be restricted to those substances not carbohydrates, proteins, fats or minerals essential to the growth and reproduction of suitable animals. The term cannot at present be justifiably extended to include substances necessary for the growth and reproduction of microörganisms and certainly not to indicate any substance serving to stimulate the growth or reproduction of microörganisms.

REFERENCES

BARTHEL, C. H. V., AND BENGTSSON, N. 1924 Action of stable manure in the decomposition of cellulose of tilled soil. Soil Sci., **8**, 185–200.

BROADHURST, JEAN 1921 The effects of vitamine extracts in revivifying old cultures. Abs. Bact., **5**, 13.

BUCHANAN, R. E. 1918 Life phases in a bacterial culture. Jour. Infect. Dis., **23**, 109–125.

CHARPENTIER, C. A. G. 1921 Studien über den Einfluss des Rindvieh- und Pferdestallmistes auf die Zersetzung der Zellulose in der Ackererde. Helsingfors.[2]

FULMER, E. I., AND NELSON, V. E. 1922 Water-soluble B and bios in yeast growth. Jour. Bio-Chem., **51**, 77–81.

FUNK, C., AND FRIEDMAN, L. 1922 Nutritional requirements in the growth of yeasts and bacteria. I. Vitamins. Jour. Metabol. Res., **1**, 457–468.

ITANO, A. 1923 Physiological study of *Azotobacter chroococcum*. I. Influence of vitamin B (?) and nucleic acid on Azotobacter. Jour. Bacteriol., **8**, 483–486.

LINOSSIER, G. 1919 Les vitamines et les champignons. Compt. rend. Soc. de biol., Par., **83**, 346–349.

LINOSSIER, G. 1919 Les vitamines et les champignons. Compt. rend. Soc. de biol., Par., **82**, 381–384.

NIKLEWSKI, B. 1912 Bodenbakteriologische Beobachtungen als Mittel zer Beurteilung von Böden. Centralbl. f. Bakteriol. (etc.), 2 Abt., **32**, 209–217.

SAMMARTINO, V. 1921 Vitamins. Biochem. Ztschr., **125**, 25; International Med. and Surg. Survey, **1-*a***, 140.

SANBORN, J. R. 1926 Physiological studies of accessory and stimulating factors in certain media. Jour. Bacteriol., **12**, 1–11.

SUZUKI, V., AND SUZUKI, B. 1923 The chemical nature of vitamin D. Jour. Chem. Soc. Japan, **44**, 228. Chem. Abs., **17**, 2907.[2]

WAKSMAN, S. A., AND SKINNER, C. E. 1926 Microörganisms concerned in the decomposition of celluloses in the soil. Jour. Bacteriol., **12**, 57–85.

WAKSMAN, S. A., AND HEUKELEKIAN, O. 1924 Microbiological analysis of soil as an index of soil fertility. VIII. Decomposition of cellulose. Soil Sci., **17**, 275–291.

WERKMAN, C. H. 1925 Continuous reproduction of microörganisms in synthetic media. Science, n.s., **62**, 114–115.

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[2] Original not available.